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PROVISIONAL APPLICATION FOR PATENT COVER SHEET
This is a request for filing a PROVISIONAL APPLICATION FOR PATENT under 37 CFR 1.53(c).

Express Mail Label No. *EV362100143US*

INVENTOR(S)		
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Additional inventors are being named on the *one* separately numbered sheets attached hereto

TITLE OF THE INVENTION (500 characters max)
Molecular Switches and Methods for Making and Using the Same Involving the Circular Permutation of DNA

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ENCLOSED APPLICATION PARTS (check all that apply)

☑ Specification *Number of Pages* __*41*__

☐ Drawing(s) *Number of Sheets* _____

☐ Application Data Sheet. See 37 CFR 1.76

☐ CD(s), Number _____

☐ Other (specify) _____

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[Page 1 of 2]

Respectfully submitted,

SIGNATURE _____

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Date *March 26, 2004*

REGISTRATION NO. *45,282*
(if appropriate)
Docket Number: *4399*

USE ONLY FOR FILING A PROVISIONAL APPLICATION FOR PATENT

PROVISIONAL APPLICATION COVER SHEET
Additional Page

	Docket Number	4399

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[Page 2 of 2]

Number _____2_____ of ___2___

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Report of Invention
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INVENTION INFORMATION

Title of Invention:

Molecular Switches and Methods for Making and Using the Same Involving the Circular Permutation of DNA

School(s) and Department(s) in which invention was developed: School of Engineering, Dept. of Chemical and Biomolecular Engineering

Additional inventors: ☒ Yes ☐ No   If yes, please complete Additional Inventors section for each inventor.

Lead Inventor Information: ["The Lead Inventor is the primary contact person for LTD on all matters associated with this Report of Invention, including processing, patent prosecution and licensing. For reasons of administrative efficiency, it is the responsibility of the Lead Inventor to keep all other JHU inventors named on this Report of Invention informed of the status of such matters."]

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INVENTION DESCRIPTION

Describe the invention completely, using the outline given below. Please provide an **Electronic Copy** of the invention disclosure document, references, and abstracts, in Windows format, on CD-Rom or Floppy Disk.

1. Abstract of the Invention [In order to assist Licensing and Technology Development with the assessment of this technology, please provide a summary of the invention that should be written to be understood by a wide audience including non-technical individuals]

The invention provides molecular switches which couple external signals to functionality and to methods of making and using the same. The switches according to the invention can be used, for example, to regulate gene transcription, target drug delivery to specific cells, transport drugs intracellularly, control drug release, provide conditionally active proteins, perform metabolic engineering, create molecular sensors, and modulate cell signaling pathways. Libraries comprising the switches and expression vectors and host cells for expressing the switches are also provided. These libraries involve the circular permutation of DNA.

2. Problem Solved [Describe the problem solved by this invention]

This invention enables the creation of fusions between nucleic acids by the insertion of one piece into another in order to functionally couple existing functionalities, modulate existing functionalities or create novel functionalities. The following list of applications that these engineered nucleic acids or proteins can have is illustrative of their broad potential: (a) regulation of gene transcription, (b) modulation of cell signaling pathways, (c) targeted drug delivery, (d) drug transport, (e) conditionally active toxic proteins, (f) metabolic engineering, (g) biosensors and (h) controlled drug release. Domain insertion is the most promising and most general strategy for engineering a molecular switch, as other strategies are limited in the sorts of signals that can be employed or the types of proteins that can be controlled. However, there are several limitations of existing domain insertion strategies that this invention overcomes: (1) they are not combinatorial. A combinatorial approach systematically tests a plurality of potential switches to find the optimum switch. (2) The switching behavior achieved by existing methods was generally modest (less than 2-fold effect). (3) None of the studies have explored circular permutation of either the target or the inserted gene; thus, existing domain insertion strategies can only explore a limited number of geometric configurations between the two domains.

3. Novelty [Identify those elements of the invention that are new when compared to the current state of the art]

To our knowledge, a circularly permuted gene that has been circularly permuted at a specific site and inserted into another gene (i.e. by domain insertion at a specific site or by random domain insertion) has never been described. To our knowledge, the insertion of a randomly circularly permuted gene into another gene (i.e. by domain insertion at a specific site or by random domain insertion) has never been described. Furthermore, the idea that such a process will create a molecular switch does not have precedent and is not obvious.

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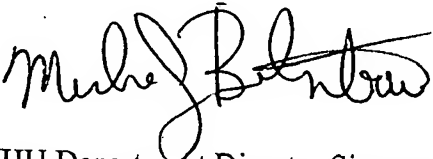
ACKNOWLEDGMENT, CERTIFICATION and ASSIGNMENT OF INVENTION

In order for this Report of Invention to be complete and processed by LTD, it must be signed and dated by:

(1)   the JHU Department Director for each JHU department involved with the development of this invention (SECTION A), and,

(2)   ALL Inventors (SECTIONS B and C), including those Inventors not subject to The Johns Hopkins University Intellectual Property Policy. Each Inventor must complete only one of Sections B or C (See explanations below).

(3)   Please duplicate Sections A, B and/or C as needed for proper completion with ALL appropriate signatures.

SECTION A. JHU DEPARTMENT DIRECTOR'S ACKNOWLEDGEMENT

I have read and understood this Report of Invention.

JHU Department Director Signature	Typed or Printed Name / JHU School / Department	Date
[signature]	*Michael Betenbaugh* Typed or Printed Name *Chem. + Biomol. Eng.* JHU School / Department	
	Typed or Printed Name JHU School / Department	
	Typed or Printed Name JHU School / Department	

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SECTION B.   JHU INVENTOR CERTIFICATION and ASSIGNMENT

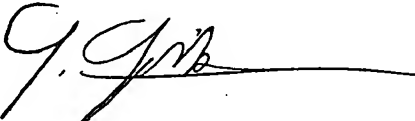This section is to be completed only by those JHU personnel subject to The Johns Hopkins University Intellectual Property Policy. Non-JHU Inventors, and HHMI or KKI Inventors at JHU are subject to a separate assignment and must complete Section C. JHU Inventors who believe they are not subject to The Johns Hopkins University Intellectual Property Policy for the invention described herein must complete Section C.

I/we, the Inventors, hereby certify that the information set forth in this Report of Invention is true and complete to the best of my/our knowledge.

I/we, the Inventors, hereby certify that I/we will promptly advise LTD of any commercial interest regarding the invention described herein.

I/we, the Inventor(s), subject to The Johns Hopkins University Intellectual Property Policy and not under an obligation to assign intellectual property rights to another party, hereby affirm that in consideration for The Johns Hopkins University's evaluation of commercial potential and a share of income which I/we may receive upon commercialization of my/our invention, on the date of my/our signature(s) as indicated below do hereby assign and transfer my/our entire right, title and interest in and to the invention described herein unto The Johns Hopkins University, its successors, legal representatives and assigns.

JHU Inventor Signature	Typed or Printed Name	Date
	MARC OSTERMEIER	
	GURKAN GUNTAS	

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Molecular Switches and Methods for Making and Using the Same Involving the Circular Permutation of DNA

Abstract

The invention provides molecular switches which couple external signals to functionality and to methods of making and using the same. The switches according to the invention can be used, for example, to regulate gene transcription, target drug delivery to specific cells, transport drugs intracellularly, control drug release, provide conditionally active proteins, perform metabolic engineering, create molecular sensors, and modulate cell signaling pathways. Libraries comprising the switches and expression vectors and host cells for expressing the switches are also provided. These libraries involve the circular permutation of DNA.

Background

Molecular Switches. A hallmark of biological systems is the high degree of interactions amongst and within their constituent components. One advantage that such interactions bring is the establishment of coupling between different functions. A protein that couples two functions can be described as a molecular switch. For example, an allosteric enzyme is a switch that couples effector levels (input) to enzyme activity (output). In most general terms, a molecular switch couples signals (e.g. ligand binding, protein-protein interactions, pH, covalent modification, temperature) to functionality (e.g. enzymatic activity, binding affinity, fluorescence). Molecular switches can be of an "on/off" nature or such that the signal modulates the function between two different levels of activity. The network of such molecular switches establishes the complex circuits that control cellular processes.

The design of molecular switches to modulate or report on biological functions has enormous potential for a variety of applications including the creation of biosensors (Siegel and Isacoff 1997; Baird, Zacharias et al. 1999; Doi and Yanagawa 1999; de Lorimier, Smith et al. 2002; Fehr, Frommer et al. 2002) modulators of gene transcription and cell signaling pathways (Rivera 1998; Guo, Zhou et al. 2000; Picard 2000), and novel biomaterials (Stayton, Shimoboji et al. 1995) (see also Figure 6). Despite their vast potential, molecular switches have not been explored extensively, in part due to the paucity of universal strategies for engineering them and the difficulty in engineering a switch that responds to a signal unrelated to the proteins' function and activates in the presence of the signal (as opposed to, for example, active-site inhibitors). In general, existing strategies for creating switches are inherently limited in the nature of the function that can be controlled, the signal that can be employed, the lack or reversibility, the lack of sensitivity, or the requirement for additional cellular components.

Domain Insertion. Gene fusion technology, the fusion of two or more genes into a single gene, has been widely used as a tool in protein engineering, localization and purification. There are two conceptually different methods of making fusions. The simplest method of end-to-end fusions has been used almost exclusively. The second method is insertional fusion in which one gene is inserted into the middle of the other gene. Insertions result in a continuous domain being split into a discontinuous domain. In nature, although continuous domains are more common, discontinuous domains are not

rare (Russell and Ponting 1998) and a systematic survey of structural domains indicated that 28% of structural domains are discontinuous (Jones, Stewart et al. 1998).

The observation of insertions in naturally occurring proteins suggests that such a route can be viable to construct proteins with desired properties and function. Furthermore, insertional fusions offer an advantage in numbers, as there are many more insertional fusions that can be made between two proteins than end-to-end fusions. Whereas it is expected that many of these insertional fusions will not be able to fold correctly, studies on insertions of DNA coding for insertions of a few amino acids to entire proteins, summarized below, have succeeded in creating active, often fully active, hybrids.

Early work on one or two codon insertions indicated that such small insertions had a smaller effect on activity than larger insertions (Boeke 1981; Stone, Atkinson et al. 1984; Barany 1985; Barany 1985; Freimuth and Ginsberg 1986; Barany 1987). Work in Paul Schimmel's lab in the late 1980's showed that insertions of random sequences into *E. coli* methionyl-tRNA synthetase of up to 14 amino acids could be tolerated in select locations (Starzyk, Burbaum et al. 1989). Subsequently other large insertions (Zebala and Barany 1991; Ladant, Glaser et al. 1992; Hallet, Sherratt et al. 1997), even a randomized 120 amino acid library insertion (Doi, Itaya et al. 1997) have confirmed this plasticity of proteins for insertions.

The first example of successful insertion of one protein into another was of alkaline phosphatase (AP) into the E. coli outer membrane protein MalF, constructed as a tool for studying membrane topology (Ehrmann, Boyd et al. 1990). High levels of alkaline phosphatase activity were obtained in the fusions despite the fact that alkaline phosphatase requires dimerization for activity. Since then, AP has been successfully inserted into a number of integral membrane proteins (Bibi and Beja 1994; Lacatena, Cellini et al. 1994; Pigeon and Silver 1994; Sarsero and Pittard 1995; Pi and Pittard 1996; Cosgriff and Pittard 1997). Furthermore, other proteins with their N- and C-termini proximal, including green fluorescent protein GFP) (Siegel and Isacoff 1997; Biondi, Baehler et al. 1998; Kratz, Bottcher et al. 1999; Siegel and Isacoff 2000), TEM1 β-lactamase (Betton, Jacob et al. 1997; Doi and Yanagawa 1999; Collinet, Herve et al. 2000), thioredoxin (Lu, Murray et al. 1995), dihydrofolate reductase (Collinet, Herve et al. 2000), FKBP12 (Tucker and Fields 2001), estrogen receptor-α (Tucker and Fields 2001) and β-xylanase (Aÿ, Götz et al. 1998), have been successfully inserted into other proteins.

Relatively few studies have examined or attempted functional coupling in insertional fusions (i.e. creating a molecular switch). Although the goal of the work was not to functionally coupled the proteins, studies of the insertion of β-lactamase into MBP (Betton, Jacob et al. 1997) and the insertion of β-lactamase or DHFR into yeast phosphoglycerate kinase (PGK) (Collinet, Herve et al. 2000) indicate that a minimal level of functional coupling of the two activities can exist, even in fusions designed to minimize interaction between the two domains. More recently, yeast sensors for ligand binding were constructed by the insertion of FKBP12 and estrogen receptor-α ligand-binding domain (EPα-LBD) into a rationally chosen site in dihydrofolate reductase (DHFR) (Tucker and Fields 2001) in the first designed coupling of growth rate of an organism to a small molecule ligand by domain insertion. Yeast expressing the FKBP12-DHFR or ERα -DHFR fusion proteins had an approximate two-fold increase in growth

rate in the presence of their respective ligands (FK106 and estrogen) when DHFR activity limited growth. However, in vitro neither the switches activity nor stability significantly changed upon ligand binding and the mechanism for increased growth rate of cells bearing these genes is not clear.

Domain insertion for has been used to couple ligand binding and changes in fluorescence. The optical signal transduction of the green fluorescent protein (GFP) has made it an attractive target for engineering biosensors by domain insertion (Guerrero and Isacoff 2001). GFP has been inserted into voltage-gated channels for potassium (Siegel and Isacoff 1997) and sodium (Ataka and Pieribone 2002) to generate sensors in which voltage driven rearrangements in the channel alter the brightness of GFP by 5.0% and 0.5% respectively. Initial attempts at creating such molecular sensors by inserting β-lactamase into GFP (Doi and Yanagawa 1999) were unsuccessful; however, random mutagenesis on the fusion was able to create a protein whose fluorescence increased 60% upon binding of the β-lactamase inhibitory protein. Insertions of calmodulin (a Ca^{2+} binding protein) into GFP resulted in a fusion whose fluorescence changed up to 40% upon increases in Ca^{2+} concentration (Baird, Zacharias et al. 1999). In a related strategy, the gene for a circularly permuted GFP was sandwiched between the gene's for calmodulin and it's target peptide M13 to create a series of sensors whose fluorescence intensity increased, decreased or showed a excitation wavelength change upon binding $Ca2^{+}$ (Nagai, Sawano et al. 2001). The GFP sensors described in this paragraph were developed through trial and error. For example, the site for inserting GFP into the sodium channel that showed a 0.5% change was the only one of the eight sites tried that showed a response (Ataka and Pieribone 2002).

Domain insertion has also been used in a strategy called mutually exclusive folding (ref) in which either one or the other of the two domains can be folded at one time. A signal (such as the presence of the ligand of one the domains) can alter the equilibrium between which domain is folded (and active). The ligand can stabilize the domain it binds to, thus causing the other domain to unfold. However, such a system is an off-switch (the signal turns the function that is to be controlled off) and thus is not as generally useful for molecular switch applications.

Random Domain Insertion. We have previously described a method for creating molecular switches for which JHU (JHU ref 1706) has filed a provisional patent (60/362,588) and a PCT application. A schematic representation of an exemplary implementation of this method is shown in Figure 1. The method creates a library of random insertions of one gene (or gene fragment) into another gene (or gene fragment). From this library molecular switches can be selected or screened for.

We demonstrated this method by creating two allosteric enzymes (i.e. enzymatic molecular switches) (Guntas and Ostermeier 2004). These allosteric enzymes have been created by the covalent linkage of non-interacting, monomeric proteins with the prerequisite effector-binding and catalytic functionalities, respectively. The fragment of the TEM-1 β-lactamase gene coding for the mature protein lacking its signal sequence was randomly inserted into the E. coli maltose binding protein (MBP) gene to create a domain insertion library. This library's diversity derived both from the site of insertion and from a distribution of tandem duplications or deletions of a portion of the MBP gene at the insertion site. From a library of ~$2x10^4$ in-frame fusions, ~800 library members conferred a phenotype to E. coli cells that was consistent with the presence of

bifunctional fusions that could hydrolyze ampicillin and transport maltose in E. coli. Partial screening of this bifunctional sublibrary resulted in the identification of two enzymes in which the presence of maltose modulated the rate of nitrocefin hydrolysis. For one of these enzymes, the presence of maltose increased k_{cat} by 70% and k_{cat}/K_m by 80% and resulted in kinetic parameters that were almost identical to TEM-1 β-lactamase. Such an increase in activity was only observed with maltooligosaccharides whose binding to MBP is known to induce a conformational change from the open to the closed form. Modulation of the rate of nitrocefin hydrolysis could be detected at maltose concentrations less than 1 μM. Intrinsic protein fluorescence studies were consistent with a conformational change being responsible for the modulation of activity.

Other strategies for creating molecular switches. There are five predominant existing strategies for creating protein molecular switches: (1) domain insertion(discussed above), (2) control of oligomerization or proximity using chemical inducers of dimerization (CID), (3) chemical rescue, (4) fusion of the target protein to a steroid binding domain (SBD) and (5) coupling proteins to nonbiological materials such as 'smart' polymers (Stayton, Shimoboji et al. 1995; Ding, Fong et al. 2001; Kyriakides, Cheung et al. 2002) or metal nanocrystals (Hamad-Schifferli, Schwartz et al. 2002). A sixth related strategy, control by regulation of aggregation (Rivera, Wang et al. 2000), is, strictly speaking, is a method for controlling a protein's availability and not a soluble protein's activity. A seventh strategy, introducing proximal cysteine residues into a protein and then modulating a protein's activity by controlling whether the cysteine forms a disulfide is obviously very limited in the signals that can be employed (ref).

Control using a chemical inducer of dimerization (CID). This strategy utilizes a synthetic ligand as the CID that controls the oligomeric or proximity of two proteins (Spencer, Wandless et al. 1993), best exemplified by signal transduction and gene expression (Rivera 1998). The CIDs are small molecules that have two binding surfaces that facilitate the dimerization of domains fused to target proteins. This was first developed using the immunosupressant FK506 to facilitate dimerization of target proteins fused to the FK506-binding protein, FKBP12 (Spencer, Wandless et al. 1993). Several variations on this system as well as a system using the antibiotic coumermycin to dimerize proteins fused to B subunit of bacterial DNA gyrase (GyrB) (Farrar, Olson et al. 2000) have appear since. CIDS have been used to initiate signaling pathways by dimerizing receptors on the cell surface, to translocate cytosolic proteins to the plasma membrane, to import and export proteins from the nucleus, to induce apoptosis and to regulate gene transcription (Bishop, Buzko et al. 2000; Farrar, Olson et al. 2000). However, CIDs have only been applied to those functions that require changes in the oligomeric state or proximity of the two proteins. As described in the literature, this approach can not be readily applied to a single protein.

Chemical Rescue. Chemical rescue is the restoration of activity to a mutant, catalytically defective enzyme by the introduction of a small molecule that has the requisite properties of the mutated residues. Since first described for subtilisin (Carter and Wells 1987), chemical rescue has been demonstrated for a number of different mutated protein-small molecule pairs (Williams, Wang et al. 2000). The vast majorities of these rescues required > 5 mM to showed detectable rescue and the maximum fold improvement in activity of the mutant was generally less than 100-fold and required >100

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mM concentrations of the rescuing molecule. Chemical rescue has only recently been applied as a strategy for control, in this case of dimerization (Guo, Zhou et al. 2000).

Fusion to a steroid binding domain. The protein to be controlled is fused end-to-end to a SBD (Picard 2000). In the absence of the steroid that binds to the SBD, it is believed that a Hsp90-SBD complex sterically interferes with the activity of the protein fused to the SBD. The disassembly of the complex upon steroid binding restores activity to the protein. This strategy has been successfully applied, principally to transcription factors and kinases (Picard 2000). Artificial transcription factors using this strategy have been developed (called GeneSwitch) and have promise for tissue-specific gene expression in transgenic animals and human gene therapy (Burcin, BW et al. 1998; Burcin, Schiedner et al. 1999).

Coupling to non-biological materials The protein to be controlled is coupled to a non-biological material that responds to an external and thereby affects the protein coupled to it. 'Smart' polymers that change their conformation upon a change in pH or temperature have been conjugated to proteins near ligand-binding sites to create switches that sterically block access to the binding site at, for example higher temperature but not at lower temperatures (Stayton, Shimoboji et al. 1995; Ding, Fong et al. 2001). Inductive coupling of a magnetic field to metal nanocrystals attached to biomolecules resulting in an increase in local temperature thereby inducing denaturation, has so far only been applied to DNA (Hamad-Schifferli, Schwartz et al. 2002) but presumably will also work with proteins.

Limitations of existing approaches. Domain insertion is the most promising and most general strategy for engineering a molecular switch, as the other strategies are limited in the sorts of signals that can be employed or the types of proteins that can be controlled. CIDs have only been applied to those functions that require changes in the oligomeric state or proximity of the two proteins and thus cannot be used to control a single protein. The serious limitation of the chemical rescue approach is the inability to apply the method to any signal and the lack of sensitivity (high concentrations of the signal are required for a small change in activity). There are several factors that limit the SBD strategy as a general method for controlling any protein. End-to-end fusion appears applicable only to fusions with SBDs as no other similar system has appeared in the thirteen years since its introduction (Picard, Salser et al. 1988; Eilers, Picard et al. 1989). Thus, the potential for the creation of molecular switches triggered by non-steroid signals by this method appears very limited. Also, a steroid is used to impart control, but steroids have pleiotropic effects and thus are not likely to be useful for engineering therapeutic molecules. Finally, the system requires a fourth component, the Hsp90 complex and apparently has low induction ratios (about ten fold) (Spencer 1996). Coupling to non-biological systems are limited in the signals that can be employed. Primarily the only signals that have been employed are pH and temperature and it is difficult to image that this strategy could be used for signals that are proteins or metabolites.

There are several limitations of existing domain insertion strategies: (1) With the exception of our work, all domain insertion studies for creating molecular switches have examined a very small number of possible insertional fusions between the two domains (i.e. the insertion locations were rationally chosen). Just as combinatorial approaches have proven to be invaluable for improving protein function by directed evolution

(Arnold 2001) (e.g. random mutagenesis and DNA shuffling), the development of combinatorial methods for domain insertion will likely have a similar effect on the development of molecular switches. (2) The switching behavior achieved my existing methods was generally modest (less than 2-fold effect). A combinatorial approach systematically tests a plurality of potential switches to find the optimum switch. (3) None of the studies have explored circular permutation of either the target or the inserted gene; thus, existing domain insertion strategies can only explore a limited number of geometric configurations between the two domains.

Circular permutation. A circularly permuted protein has its original N- and C-termini fused and new N- and C- termini created by a break elsewhere in the sequence. The first *in vitro* construction of a circular permuted protein was carried out on bovine pancreatic trypsin inhibitor by chemical means (Goldenberg and Creighton 1983). Since then, a number of circular permuted proteins have been constructed (Heinemann and Hahn 1995), primarily by genetic methods, including TEM-1 β-lactamase (Osuna, Pérez-Blancas et al. 2002) used in this study. These studies have shown that circular permuted proteins very often fold up into stable, active proteins. Comparisons of primary and tertiary structures within several protein families have led to the conclusion that circular permutation occurs in natural protein sequences.

Random circular permutation. A genetic method for *random* circular permutation of any gene was first described by Graf and Schachmann (Graf and Schachman 1996). The method is comprised of the following steps: (i) isolation of a linear fragment of double stranded DNA (of the gene to be randomly circularly permuted) with flanking compatible ends, (ii) cyclization of this DNA fragment by ligation under dilute conditions, (iii) random linearization of the cyclized gene using DNase I digestion in the presence of Mn^{2+} at dilute concentrations of the enzyme such that the DNase I, on average, makes one double strand break, (iv) repair of nicks and gaps using a DNA polymerase and a DNA ligase, and (v) ligation of the fragment into a desired vector by blunt end ligation to create the plasmid library of randomly circularly permuted genes. The procedure has been used to systematically identify permissive sites for circular permutation in aspartate transcarbamoylase (Graf and Schachman 1996), DsbA (Hennecke, Sebbel et al. 1999) and GFP (Baird, Zacharias et al. 1999) and DHFR (Iwakura, Nakamura et al. 2000) and in most case a variety of permissible sites were identified. The original random circular permutation protocol (ref) as published works poorly, if at all and likely had errors in it (Graf and Schachman 1996). The methodology for randomly circularly permuting DNA was improved upon in a study involving the random localization of a restriction enzyme site in a fixed length of DNA (Ostermeier and Benkovic 2001).

Novelty. To our knowledge, a circularly permuted gene that has been circularly permuted at a specific site and inserted into another gene (i.e. by domain insertion at a specific site or by random domain insertion) has never been described, though such a construct has been proposed (but not tested) as having potential for creating GFP sensors (Baird, Zacharias et al. 1999). To our knowledge, the insertion of a randomly circularly permuted gene into another gene (i.e. by domain insertion at a specific site or by random domain insertion) has never been described. Furthermore, the idea that such a process will create a molecular switch does not have precedent and is not obvious.

Description of the Invention

This invention combines circular permutation and domain insertion in a novel fashion to create molecular switches. The invention is reduced to practice using TEM-1 β-lactamase (BLA) and *E. coli* maltose binding protein (MBP) as model proteins. These are the same proteins used to demonstrate how random domain insertion alone can create switches (our previous invention). Although the random domain insertion method alone has been able to create a BLA-MBP molecular switch, to date the magnitude of the switching achieved has been modest (< 2-fold) and only "on-switches" were found (i.e. the addition of signal resulted in an increase in function). The switching obtained using circular permutation and domain insertion has resulted in both "on" and "off" switches and magnitude of the switching has been much higher (in one case, at least 32-fold). Although demonstrated with two particular proteins this invention applies to functionally coupling any two proteins.

Key to this invention is the circular permutation of one of the genes (in this case the insert gene, although circular permutation of the acceptor sequence is also possible). A β-lactamase that has been circularly permuted and inserted into MBP will have a different covalent linkage and a different spatial orientation to the MBP compared to the original insertion using the wildtype N- and C-termini of BLA. This potentially can have dramatic effects on the switching behavior. For example, the switching behavior can change due to (1) decreasing the distance from the active site of β-lactamase to residues allosterically linked to maltose binding to MBP, (2) exploring new paths by which the effects of maltose binding can propagate to the active site of β-lactamase and (3) changing the stability of the BLA domain making it more susceptible to changes in conformation of the MBP domain. For example, the switches identified by domain insertion of a non-circularly permuted BLA (Guntas and Ostermeier 2004) were linked through a very stable portion of the β-lactamase (Luque and Freire 2000). Circularly permuting β-lactamase will allow linkage through less stable regions that are postulated to be more sensitive to the effects of maltose binding.

Two strategies for creating molecular switches involving random circular permutation of the insert gene have been demonstrated (Figure 2) using MBP and BLA as an example. In the first, (called "Random Circular Permutation of Insert and Domain Insertion at a Specific Site") the BLA gene is circularly permuted and inserted into a specific site in the MBP. This site could be a site previously shown to be useful for creating molecular switches (as is demonstrated here) or a site that is predicted, by computational methods or other means, to be useful in creating a molecular. In the second method (called "Random Circular Permutation of Insert and Random Domain Insertion") the BLA gene is randomly circularly permuted and randomly inserted into a plasmid containing the MBP gene. The use of BLA and MBP are only examples and potentially any two proteins can be functionally coupled in this manner.

Linkers for Circular Permutation. In order to circularly permute a gene it is usually necessary to include DNA that codes for a linker to link the original N- and C-termini. We chose to test two different linkers. In the first (the "DKS linker") the β-lactamase was randomly circularly permuted by fusing the 5'- and 3'- ends with a DNA sequence coding for the tripeptide linker DKS previously found in a combinatorial library of linkers to be most conducive for circularly permuting β-lactamase when the new N- and C-termini were located at a specific location (Osuna, Pérez-Blancas et al. 2002). In

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the second (the "GSGGG linker") the β-lactamase was randomly circularly permuted by fusing the 5'- and 3'- ends with a DNA sequence coding for the flexible pentapeptide linker GSGGG.

Preparation of BLA Insert DNA. The β-lactamase gene fragment *bla [24-286]* (codes for amino acids 24-286) was amplified by PCR from pBR322 such that it was flanked by *Ear*I or *Bam*HI sites and sequences coding for the linkers described above and cloned into pGem T-vector (Promega) to create pBLA-CP(DKS) and pBLA-CP(GSGGG), respectively (Figures 3 and 4). DNA coding for amino acids 1-23 were not desired because it codes for the signal sequence that targets ß-lactamase to the periplasm. This sequence gets cleaved upon entering the periplasm and is not part of the mature, active ß-lactamase.

One hundred and thirty micrograms of pBLA-CP(GSGGG) was digested with 2000 units of *Bam*HI and 140 micrograms of pBLA-CP(DKS) was digested with 600 units of *Ear*I in the buffers and conditions recommended by the manufacturer of the restriction enzyme. The fragment containing the BLA gene was purified by agarose gel electrophoresis using the QIAquick gel purification kit. This DNA was treated with T4 DNA ligase under dilute concentrations to cyclize the DNA (18 hours at 16 °C with 600 Weiss units of T4 DNA Ligase in the presence of 50 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 10 mM dithiothreitol, 1 mM ATP, 25 ug/ml BSA in a total volume of 5.1 ml). The ligation reaction was stopped by incubation at 65 °C for 20 minutes. The DNA was concentrated by vacufuge and desalted using the QIAquick PCR purification kit. The circular fragment was purified by agarose gel electrophoresis using the QIAquick gel purification kit.

The conditions for DNaseI digestion were determined experimentally by adding different amounts of DNaseI and analyzing the digested products by agarose gel electrophoresis. The digestion conditions were chosen such that a significant fraction of DNA was undigested in order maximize the amount of linear DNA that only had one double strand break. In general, approximately 1 milliunit of DNaseI per microgram of DNA (at a concentration 10 micrograms/ml) of for an 8 minute digestion at 22 °C is close to being optimum. Sometimes more or less DNaseI was required and thus it is recommended that for each library constructed the correct amount of DNaseI be determined experimentally by test digestions. The following conditions are given as a representative example. Six micrograms of circular DNA was digested with 6 milliunits of DNase I (Roche) for 8 minutes at 22 °C in the presence of 50 mM TrisHCl (pH 7.4), 1 mM MnCl$_2$ and 50 micrograms/ml BSA in 0.6 ml reaction volume. The reaction was stopped by adding EDTA to a concentration of 5 mM. The DNA was desalted using the QIAquick PCR purification kit and repaired by 6 units of T4 DNA polymerase and 6 Weiss Units of T4 DNA ligase at 12 °C for 15 minutes in the presence of 100 micromolar dNTP, 50 mM Tris-HCl (pH 7.5), 10 mM MgCl$_2$, 10 mM dithiothreitol, 1 mM ATP and 25 ug/ml BSA. The repaired, linear DNA was purified by agarose gel electrophoresis using the QIAquick gel purification kit. This is the circularly permuted gene that is ready for insert into another plasmid.

Preparation of target DNA for random domain insertion libraries. Forty μg of pDIM-C8-Mal was digested with DNaseI (0.01 units) for 8 minutes at 22°C in the presence of 50 mM Tris-HCl, pH 7.4, 10 mM MnCl$_2$ and 50 μg/ml BSA in a total volume of 1 ml. The reaction was quenched by the addition of EDTA to a concentration of 5 mM

and the solution was desalted using four Qiaquick PCR purification columns into 200 μl elution buffer which was subsequently concentrated by vacufuge. Nicks and gaps were repaired by incubating at 12°C for 1 hour in a total volume of 120 μl in the presence of T4 DNA polymerase (15 units) and T4 DNA ligase (12 Weiss units) in the presence of 50 mM Tris-HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM DTT, 1 mM ATP, 25 μg/ml BSA and 125 μM dNTPs. The reaction was stopped by incubating at 80°C for 10 minutes. Sodium chloride was added to 100 mM and the DNA was dephosphorylated by adding alkaline phosphatase (60 units) and incubating at 37°C for 1 hour. The DNA was desalted as before and the linear DNA (corresponding to the randomly linearized pDIM-C8-Mal) was isolated from circular forms of the plasmid by agarose gel electrophoresis using the Qiaquick gel purification kit.

Preparation of target DNA for site-specific insertion libraries. Plasmid pDIM-C8-Mal was modified using overlap extension (Horton, Hunt et al. 1989) to be suitable for insertion of the circularly permuted BLA at two specific sites: (a) between MBP [1-165] and MBP [164-370] and (b) at the C-terminus of MBP. The plasmids were modified in analogous ways, the modifications for insertion between MBP [1-165] and MBP [164-370] to create plasmid pDIMC8-MBP(164-165) is described as an example (Figure 5). Two inverted *Sap*I sites were inserted between DNA coding for MBP [1-165] and MBP [164-370] in such a manner that digestion with *Sap*I and subsequent filling in of the resulting overhangs using Klenow polymerase in the presence of dNTPs results in a perfectly blunt *MBP [1-165]* on one side and a perfectly blunt *MBP [164-370]* on the other. This is achieved by virtue of the fact that *Sap*I is a type IIS restriction enzyme that cuts outside of its recognition sequence. Other type IIS restriction enzymes could have been used. Non-type IIS restriction enzymes could also be used if it is acceptable to have their recognition site as part of the gene fragment that is being inserted into.

Three micrograms of pDIMC8-MBP(164-165) was digested with 6 units of *Sap*I at 37 °C in the presence of 50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol (pH 7.9), 100 ug/ml BSA for 2.5 hours. The DNA was desalted using the QIAquick PCR purification kit and repaired with 5 units of Klenow at 25 °C for 20 minutes in the presence of 33 micromolar dNTPs, 100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$ and 1 mM dithiothreitol (pH 7.9). The enzyme was heat inactivated by incubation at 75 °C for 20 minutes. Sodium chloride was added to 100 mM and ten units of Calf Intestinal Phosphatase was added and the solution was incubated for 1 hour at 37 °C. Dephosphorylation was performed to prevent recircularization of the vector without receiving an insert in the subsequent ligation step. The vector DNA was purified by agarose gel electrophoresis using the QIAquick gel purification kit.

Ligation of inserts into target DNA. Insert DNA (85 ng) comprising the circularly permuted BLA was ligated to the prepared target DNA (100 ng) at 22°C overnight in the presence of T4 DNA ligase (30 Weiss units) and the ligase buffer provided by the manufacturer in a total volume of 13 μl. After ethanol precipitation, 10% of the ligase-treated DNA was electroporated into 50 μl Electromax DH5α-E electrocompetent cells. Transformed cells were plated on large (248 mm x 248 mm) LB agar plate supplemented with 50 μg/ml chloramphenicol (Cm). The naïve domain insertion library was recovered from the large plate (Ostermeier, Nixon et al. 1999) and stored in frozen aliquots.

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Screening for allosteric enzymes. The libraries were diluted from frozen aliquots and plated on LB plates containing different concentrations of ampicillin (Tables 1 and 2). A number of colonies were picked (Table 4) and grown in LB overnight in 96 well plates (0.5 ml/well) in the presence of 1 mM IPTG and 50 μg/ml Cm. Next, 50 μl of PopCulture (Novagen) and 2.5 unit of benzonase nuclease was added to each well and incubated for 15 minutes at room temperature to lyse the cells. The cells debris and any unlysed cells were pelleted by centrifugation and supernatant was recovered. In 96-well format, 60 μl of lysate was assayed for hydrolysis of nitrocefin (50 μM) by monitoring the increase in absorbance at 490 nm in 100 mM sodium phosphate buffer, pH 7.0, both with and without 5 mM maltose. Any lysate in which there was a difference in rate of more than 2-fold (between with and without maltose) was selected for retesting and further investigation.

Confirmation and identification of positives. Library members identified as having more than 200% switching activity in the 96-well plate screen were grown 24-48 hours in 100 ml LB media in 500 ml shake flasks at 22°C without IPTG. The cells were pelleted and resuspended in 8 ml assay buffer (100 mM sodium phosphate buffer, pH 7.0) and lysed by French press. The soluble fraction of this lysate was assayed for hydrolysis of nitrocefin (50 μM) at 22 °C as previously described (Guntas and Ostermeier 2004) both with and without 5 mM maltose. Initial rates were determined from absorbance at 486 nm monitored as a function of time. The enzyme was incubated at the assay temperature in the absence or presence of 5 mM maltose for four minutes prior to performing the assay. All assays contained 100 mM sodium phosphate buffer, pH 7.0. Library members for which there was a difference in the initial rate of more than about 2-fold were sequenced (Table 4). Switches RG-5-169 and RG-200-13 were also assayed in the presence of 5 mM sucrose or 5 mM glucose. Neither sugar affected the velocity of nitrocefin hydrolysis indicating that the switching effect was specific for maltose, a ligand to which MBP binds.

Analysis of Purified Switch RG-200-13. A 6xHis tag was added to the C-terminus of RG-200-13 and the fusion was purified as previously described for another switch (Guntas and Ostermeier 2004). The protein was purified to approximately 60% purity. The kinetic constants and binding constants were determined from Eadie-Hofstee plots and Eadie plot equivalents, respectively, using a spectrophotometric assay for nitrocefin hydrolysis. Initial rates for nitrocefin hydrolysis were determined from absorbance at 486 nm monitored as a function of time. The enzyme was incubated at the assay temperature in the absence or presence of saccharide for four minutes prior to performing the assay. All assays contained 100 mM sodium phosphate buffer, pH 7.0. The dissociation constant for maltose was determined using change in velocity of nitrocefin hydrolysis as a signal.

Only sugars known to bind to MBP had an effect on nitrocefin hydrolysis (Table 5). Those sugars that produce a large conformational change upon binding MBP (Quiocho, Spurlino et al. 1997) (maltose and maltotriose) produced the largest change in the velocity of nitrocefin hydrolysis. Beta-cyclodextrin, which produces a small conformational change upon binding MBP (Evenas, Tugarinov et al. 2001), has a small effect. The effect of maltotetraitol is intermediate, consistent with the fact that maltotetraitol-binding to MBP results in a mixture of open and closed structures (Duan, Hall et al. 2001).

The kinetic parameters of RG-200-13 are reported in Table 6. The kinetic parameters of RG-200-13 at 22°C in the presence of maltose ($k_{cat} = \sim520$ s^{-1}; $K_m = \sim85$ μM) are very similar to previously reported values for TEM-1 β-lactamase at 30 °C ($k_{cat} = 930$ s^{-1}; $K_m = 52$ μM) (Raquet, Lamotte-Brasseur et al. 1994) indicating that RG-200-13 is essentially a fully functional TEM-1 β-lactamase in the presence of maltose. The k_{cat}/K_m in the presence of 5 mM maltose is approximately 25-fold higher than in the absence of maltose. The K_d for maltose binding to RG-200-13 at 22°C was ~5 μM, similar to the K_d previously reported for maltose binding to MBP (1-1.5 μM) (Schwartz, Kellermann et al. 1976).

The effect of 5 mM maltose on other sustrates of BLA is shown in Table 7. Maltose binding had the largest effect on cephalothin (of the substrates tested), with the velocity of cephalothin hydrolysis being 32-fold higher in the presence of maltose than in its absence. Based on the effects on other substrates, the actual switching effect on k_{cat}/K_m for cephalothin is likely to be much higher than 32-fold.

The fact that the magnitude of the switching effect of RG-200-13 is substrate identity and concentration dependent strongly argues that maltose is converting the protein from a less active to a more active conformation. The alternative explanation that maltose affects the equilibrium between unfolded (inactive) and folded (active) forms of the protein would result in a switching effect that was independent of the substrate being tested (and independent of substrate concentration), which is not the case.

Applications of the molecular switches obtained by the invention. Protein molecular switches are proteins whose activity can be modulated through a signal such as the binding of a small molecule, the interaction with another protein or the sensing of some other signal (e.g. a change in pH). In other words, a molecular switch functionally couples an external signal to functionality. Protein molecular switches engineered by the methods of this invention have a wide variety of potential applications (Fig 6) in, for example, (a) the regulation of gene transcription, (b) the modulation of cell signaling pathways, (c) targeted drug delivery, (d) drug transport, (e) the creation of conditionally active toxic proteins, (f) metabolic engineering, and (g) the creation of novel biosensors.

The BLA-MBP switches have potential as in vitro or in vivo molecular sensors. BLA activity can be measure *in vivo* or *in vitro* using specially designed fluorescent substrate (Zlokarnik, Negulescu et al. 1998; Gao, Xing et al. 2003). MBP is a member of a class of proteins called periplasmic binding proteins that have been shown to be amenable to computational design to alter the proteins so that they bind new ligands (Looger, Dwyer et al. 2003). Thus, the BLA-MBP switches potentially can be used to detect different ligands *in vivo* as enzymatic activity would be proportional to ligand concentration.

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Table 1 Library statistics

Insertion site in MBP	Linker in BLA	Library size (Number of transformants with BLA insert).	Number of library members that can grow on 50 μg/ml AMP (see Table 2)	Number of colonies screened for switching (see Table 3)	Number of unique switches found with ≥ 2-fold effect*	Increase in velocity (of nitrocefin hydrolysis in presence of maltose) of best switch
164-165	DKS	0.44x10^6	515	848	2	+97%
	GSGGG	1.05x10^6	361	1248	1	-250%
C-terminus	DKS	1.03x10^6	2414	576	0	
	GSGGG	0.30x10^6	1615	1920	1-4	+234%
random	DKS	0.41x10^6	191	384	0	
	GSGGG	1.20x10^6	1156	3312	5	+1650%

* ≥ 2-fold change in velocity of nitrocefin hydrolysis in the presence of 5 mM maltose.

Table 2 Number of Library members that could grow on plates with ampicillin (with or without maltose)

Ampicillin (µg/ml)	Maltose? (5 mM)	T164-165 DKS	T164-165 GSGGG	EE DKS	EE GSGGG	Random DKS	Random GSGGG
5	no	734	878	7052	3510	nd	2458
50	no	394	294	1747	1159	nd	783
200	no	220	nd	1080	298	nd	nd
1000	no	nd	74	nd	nd	nd	60
5	yes	1098	761	8354	4056	nd	1969
50	yes	515	361	2414	1615	191	1156
200	yes	182	240	1525	630	nd	272
1000	yes	nd	88	nd	nd	nd	34

EE = end-to-end (insertion at C-terminus); nd = not determined

Table 3 Number of library members screened (picked from plates with indicated ampicillin and maltose levels)

Ampicillin (µg/ml)	Maltose? (5 mM)	T164-165 DKS	T164-165 GSGGG	EE DKS	EE GSGGG	Random DKS	Random GSGGG
5	no	-	96	-	288	-	96
50	no	-	-	-	-	-	-
200	no	-	-	-	-	-	480
1000	no	-	-	-	-	-	-
5	yes	96	192	-	864	-	768
50	yes	672	576	576	768	384	960
200	yes	80	384	-	-	-	1008
1000	yes	-	-	-	-	-	-

EE = end-to-end (insertion at C-terminus)

Table 4. Selected BLA-MBP Molecular Switches

Switch	Sequence	Switching effect*
IFG-5-277	MBP[1-165]-BLA[218-286]-GSGGG-BLA[24-215]-MBP[164-370]	-250%
IFD-5-7	MBP[1-165]-BLA[110-286]-DKS-BLA[24-107]-MBP[164-370]	+96%
IFD-5-15	MBP[1-165]-BLA[168-286]-DKS-BLA[24-170]-MBP[164-370]	+97%
EEG-50-530	MBP[1-370]-BLA[114-286]-GSGGG-BLA[24-112]-GSQQH	+228%
EEG-50-251	MBP[1-370]-BLA[114-286]-GSGGG-BLA[24-114]-K	+234%
RG-200-151	MBP[1-341]-A-BLA[111-286]-GSGGG-BLA[24-115]-MBP[347-370]	+350%
RG-200-604	MBP[1-331]-BLA[113-286]-GSGGG-BLA[24-112]-L-MBP[333-370]	+390%
RG-5-169	MBP[1-338]-BLA[34-286]-GSGGG-BLA[24-29]-MBP[337-370]	+855%
RG-200-13	MBP[1-316]-BLA[227-286]-GSGGG-BLA[24-226]-S-MBP[319-370]	+1650%

* Percent change in velocity of nitrocefin hydrolysis (50 µM nitrocefin) in the presence of 5 mM maltose in 100 mM sodium phosphate buffer, pH 7.0.

Table 5. Sugar dependence of switching effect of RG-200-13*.

Sugar	Binds to MBP?	Change in velocity of nitrocefin hydrolysis in presence of sugar
Sucrose	No	-5%
Lactose	No	-4%
Galactose	No	-3%
Maltose	Yes	+1800%
Maltotriose	Yes	+1700%
Maltotetraitol	Yes	+400%
β-cyclodextrin	Yes	+150%

*50 µM nitrocefin, 100 mM sodium phosphate buffer, pH 7.0, 22°C, 5 mM sugar except for β-cyclodextrin (3mM).

Table 6 Kinetic parameters of nitrocefin hydrolysis of RG-200-13 molecular switch.

Substrate	k_{cat} (s^{-1})			K_m (μM)			k_{cat}/K_m Ratio[a]
	No maltose	5 mM maltose	Ratio[a]	No maltose	5 mM maltose	Ratio[a]	
nitrocefin	~80	~520	~6.5	~325	~85	~0.26	~25

[a](with maltose)/(without maltose). Conditions: 100 mM sodium phosphate buffer, pH 7.0, 22°C.

Table 7. Effect of maltose on other substrates of switch RG-200-13

Substrate	Substrate concentration	K_m for TEM-1 β-lactamase[a]	Approximate fold increase in velocity of nitrocefin hydrolysis in the presence of 5 mM maltose
cephalothin	250 μM	246 μM	32
ampicillin	100 μM	32 μM	26
	500 μM		10
benzylpenicillin	100 μM	19 μM	17
	500 μM		7
carbenicillin	1 mM	?	4
oxacillin	1 mM	3 μM	5

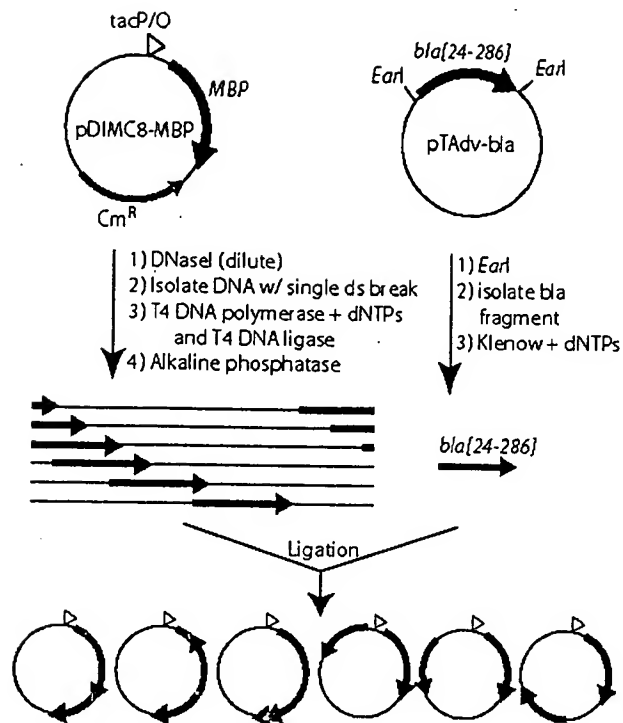Conditions:100 mM sodium phosphate buffer, pH 7.0, 22°C. [a](Raquet, Lamotte-Brasseur et al. 1994)

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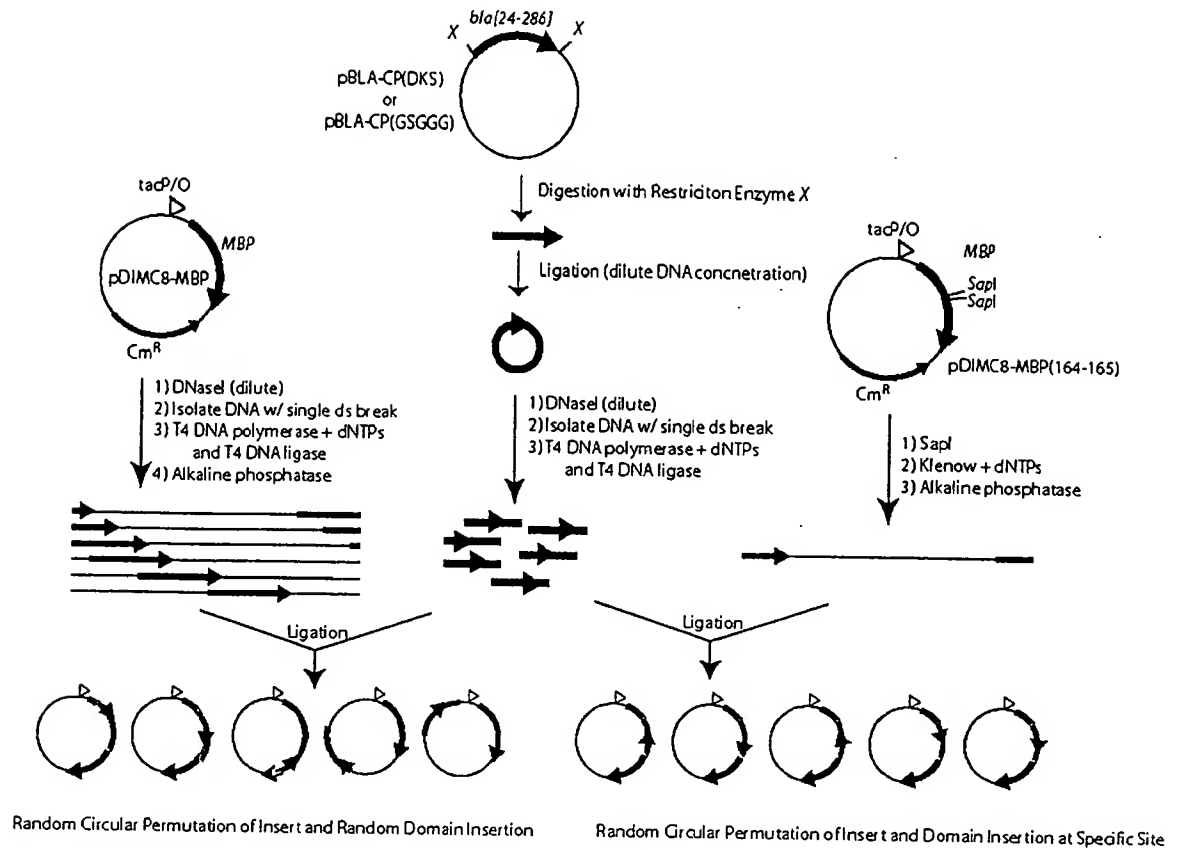Figure 1 Random Domain Insertion.

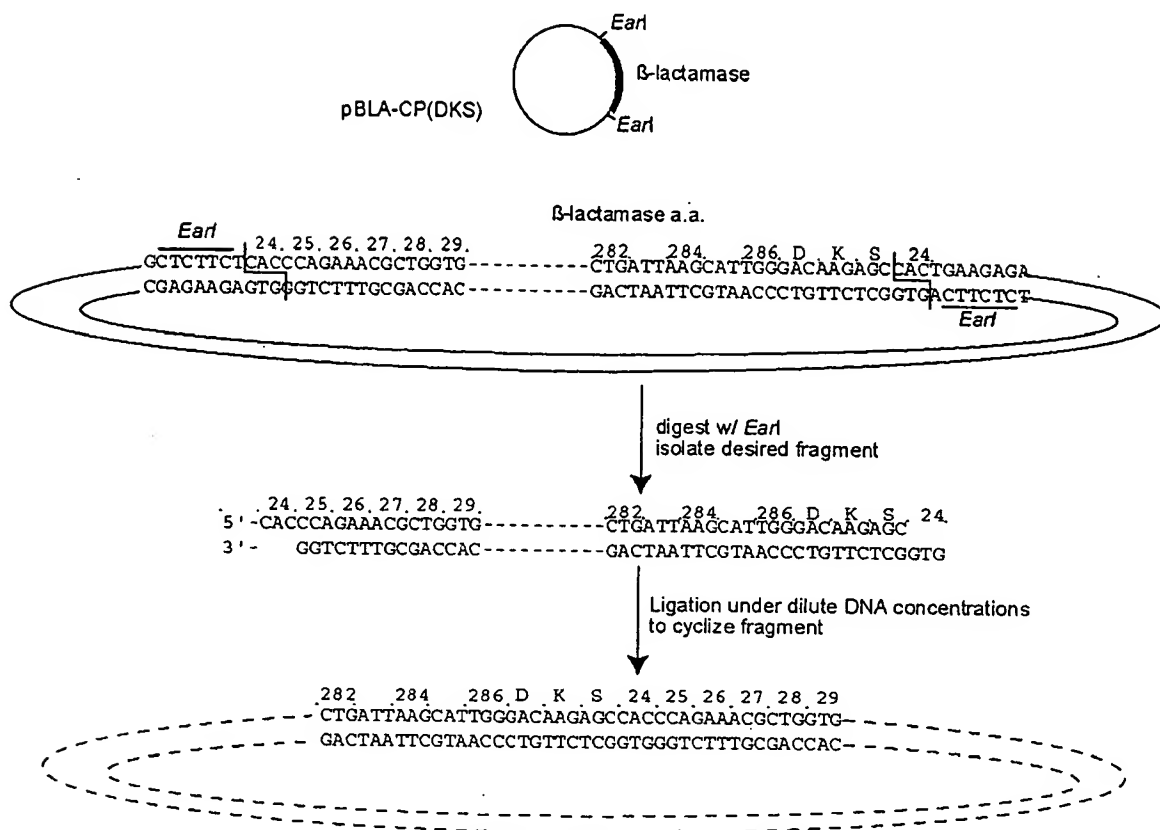Figure 2 Creation of domain insertion libraries involving the random circular permutation of DNA.

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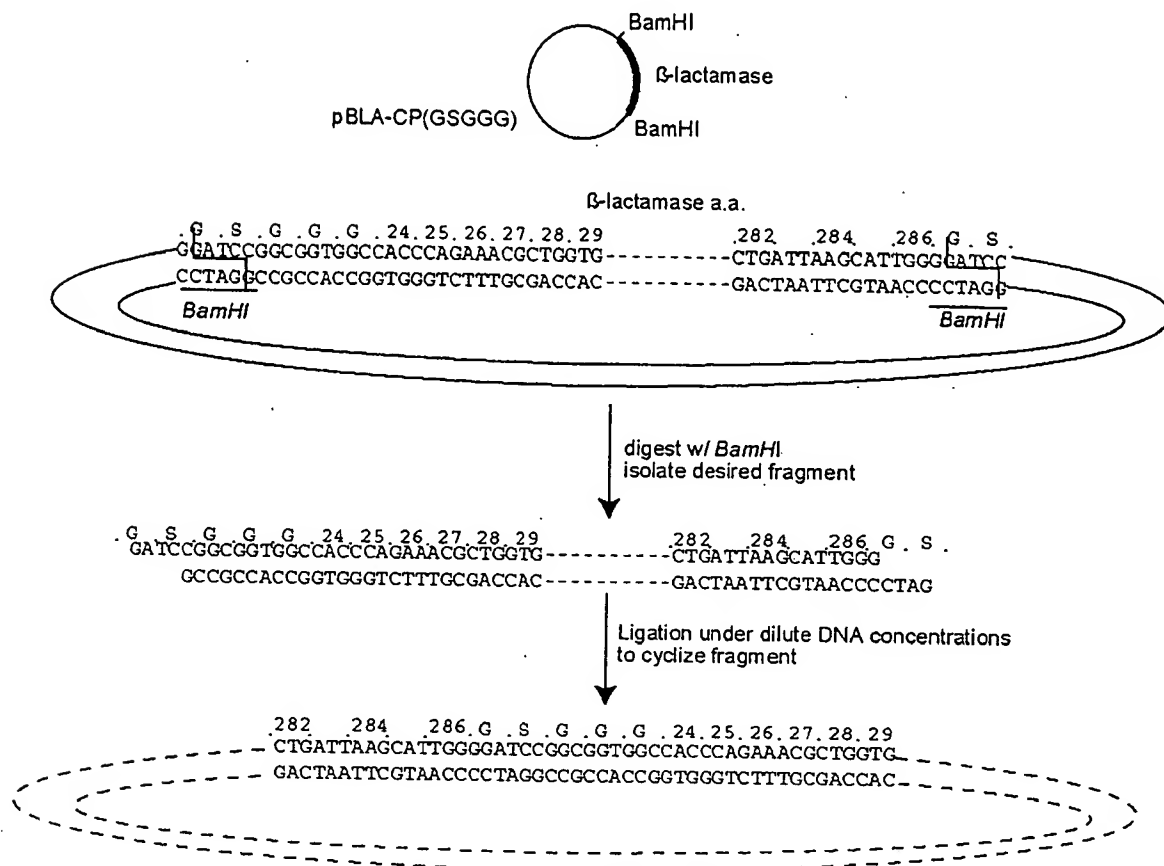Figure 3 Creation of a cyclized beta-lactamase gene with a DKS linker.

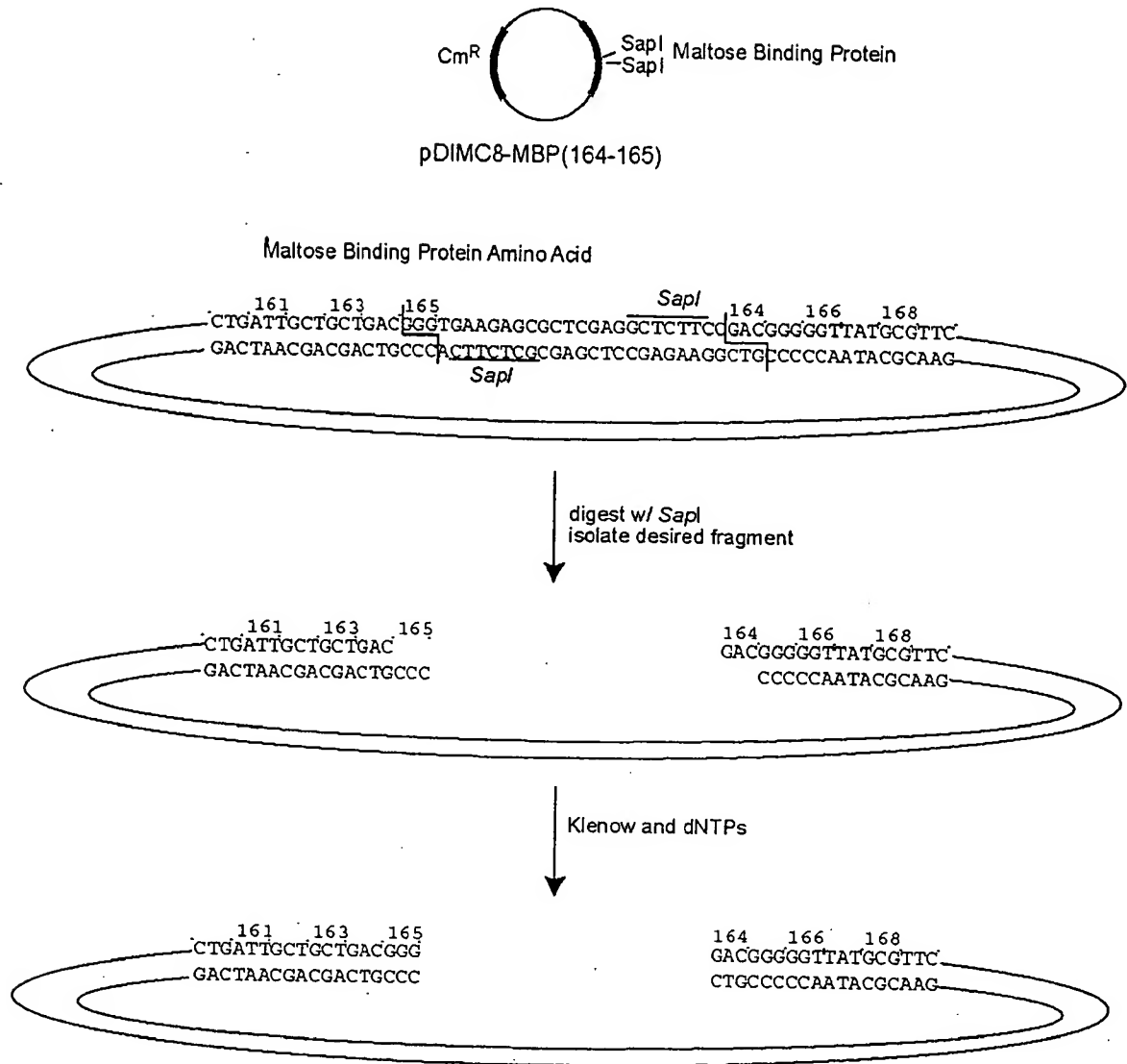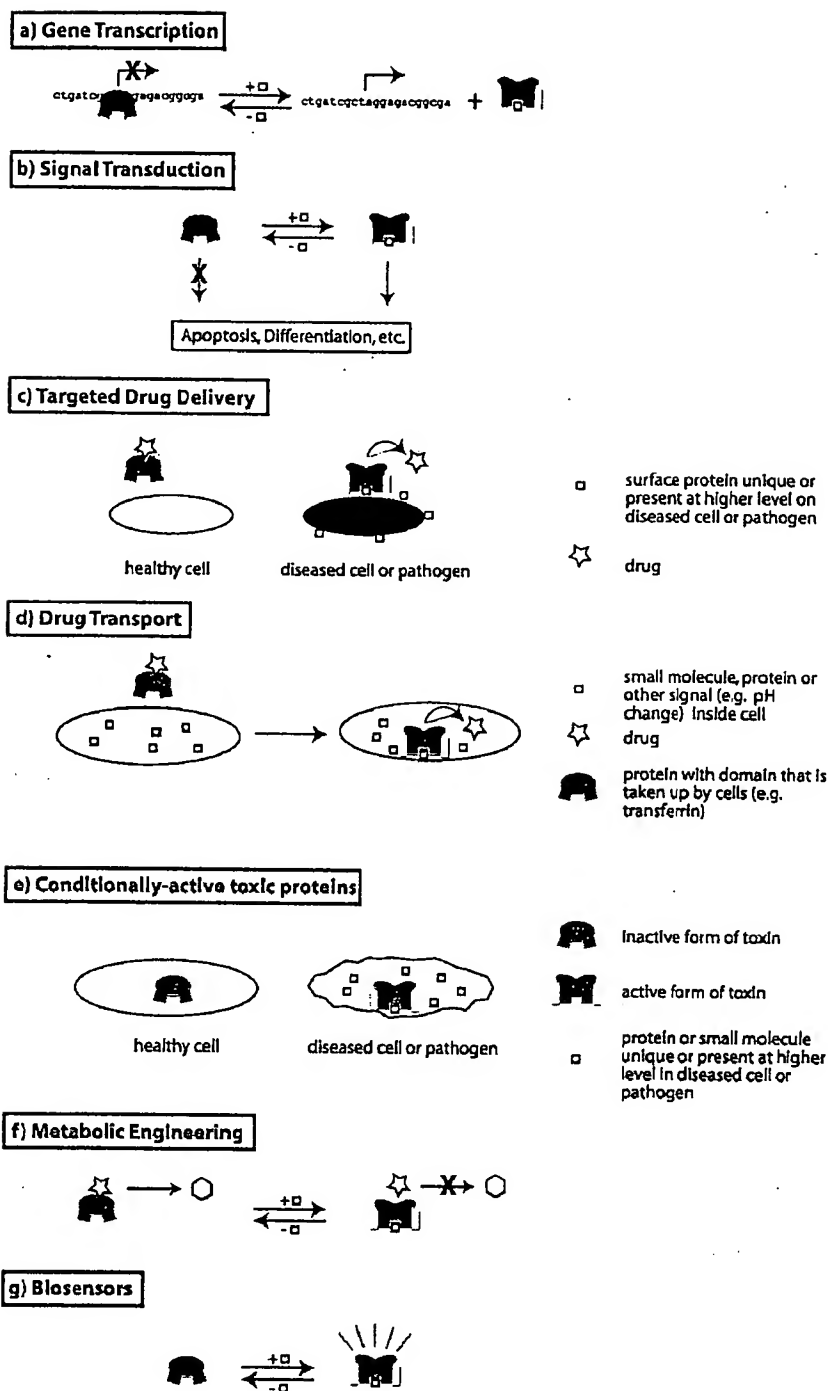Figure 4 Creation of a cyclized beta-lactamase gene with a GSGGG linker.

pDIMC8-MBP(164-165)

Maltose Binding Protein Amino Acid

Figure 5. Prepartion of target DNA for insertion of DNA at a specific location (in this case between MBP [1-165] and MBP [164-370])

a) Gene Transcription

b) Signal Transduction

Apoptosis, Differentiation, etc.

c) Targeted Drug Delivery

healthy cell   diseased cell or pathogen

□   surface protein unique or present at higher level on diseased cell or pathogen

☆   drug

d) Drug Transport

□   small molecule, protein or other signal (e.g. pH change) inside cell

☆   drug

protein with domain that is taken up by cells (e.g. transferrin)

e) Conditionally-active toxic proteins

Inactive form of toxin

active form of toxin

healthy cell   diseased cell or pathogen

□   protein or small molecule unique or present at higher level in diseased cell or pathogen

f) Metabolic Engineering

g) Biosensors

Figure 6 Application of Molecular Switches. (a) Regulation of gene transcription. A DNA binding protein is functionally coupled to a protein that binds □ such that the protein releases from the DNA in the presence of □ . The signaling molecule □ , which could be a naturally occurring in the cell or supplied as a drug, could then be used to induce expression of an exogenously supplied therapeutic or toxic protein or to repress/induce expression of endogenous genes in order to restore normal cell function. (b) Modulation of cell signaling pathways. Cell fates can be regulated by engineering a protein involved in a cell signaling pathways to be conditionally active in the presence of the signal □ , which could be a naturally occurring small molecule, a

protein or an administered drug. (c) Targeted drug delivery. A drug binding protein can be functionally coupled to a protein that binds some small molecule or protein that is a signature of a disease (e.g. a surface protein). The drug binding protein releases the drug only in the presence of the molecular signature of the disease. (d) Drug transport. A drug binding protein can be fused to a protein that is taken up by cells in order to transport the drug into cells. The two proteins are functionally coupled such that the conformational change in the transport protein upon interaction with ☐ inside the cell is coupled to the release of the drug. (e) *Conditionally active toxic proteins*. A toxic protein is engineered such that it is nontoxic unless it interacts with ☐ , which is a small molecule or protein signature of a diseased cell or pathogen. (f) *Metabolic engineering*. Just as small molecules in cells can allosterically modulate metabolic pathways, engineered molecular switches can control metabolic pathways. This could be achieved by functional coupling of an enzyme (whose activity is to be modulated) with a protein that binds ☐ . (g) *Biosensors*. The key design issue in biosensors is the creation of a macromolecule that specifically binds its target ligand and, upon binding, transduces this into a signal (such as optical or electrochemical changes) that can be detected macroscopically. The functional coupling of a binding protein and a signal transducing properties is a general strategy towards engineering new families of biosensors.

The combinatorial design of protein molecular switches

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A hallmark of biological systems is the high degree of interactions amongst and within their constituent components. One advantage that such interactions bring is the establishment of coupling between different functions. A protein that couples two functions can be described as a molecular switch. For example, an allosteric enzyme is a switch that couples effector levels (input) to enzyme activity (output). In most general terms, a molecular switch couples signals (e.g. ligand binding, protein-protein interactions, pH, covalent modification, temperature) to functionality (e.g. enzymatic activity, binding affinity, fluorescence). Molecular switches can be of an "on/off" nature or such that the signal modulates the function between two different levels of activity. The network of such molecular switches establishes the complex circuits that control cellular processes. Protein molecular switches have a wide variety of potential applications including the regulation of gene transcription, the modulation of cell signaling pathways, targeted drug delivery, drug transport, the creation of conditionally active toxic proteins, metabolic engineering, and the creation of novel biosensors. We have devised a combinatorial protein engineering algorithm that can create molecular switches by the coupling of two proteins' functionalities. We have demonstrated the efficacy of the algorithm by creating a set of allosteric enzymes from two unrelated proteins with the prerequisite effector-binding and catalytic functionalities, respectively. In some of these engineered molecular switches, the presence of effector increased catalytic activity by more than ten-fold.

Described are methods for creating and using molecular switches with different affinities for the effector molecule (signal) as well as methods for creating and using molecular switches with different specificities for the effector molecule (i.e. it responds to a new signal).

We have demonstrated a method for creating a molecular switch by a combinatorial approach of recombining two genes so as to couple their functions. We have demonstrated the approach by recombining the genes for TEM1 β-lactamase (BLA) and the *E. coli* maltose binding protein (MBP) such that BLA catalytic activity was a function of the concentration of maltose. The kinetic parameters and affinity for maltose of one of these switches (RG13 –referred to in the previous provisional as RG-200-13 – which has the amino acid sequence MBP[1-316]-BLA[227-286-GSGGG-BLA[24-226-S-MBP[319-370]) have been determined and are shown in Tables 1 and 2. RG13 has a dissociation constant for maltose is about 5-6 μM in the absence of a BLA substrate. In the presence of saturating amounts of the substrate carbenicillin, the dissociation constant for maltose decreases to about 1 μM. This shows that the binding of maltose and substrate (carbenicillin) are coupled. The coupling energy is on the order of 1 kcal/mol. This is consistent with a decrease in K_m for nitrocefin in the presence of maltose (Table 2)

A switch is most useful if the range of the concentration of the signal (maltose, in the case of RG13) overlaps with the range of concentration of the signal that the dependent function responds to. For the case when a ligand-binding protein is used as the signal detector and the ligand is the signal, the later range corresponds to approximately to the range $0.1K_d - 10\ K_d$, where K_d is the dissociation constant of the switch and the signal. This can be seen from the following example:

In the case of RG13, the velocity of nitrocefin hydrolysis is the dependent function. The velocity (v) of nitrocefin hydrolysis depends on the steady state kinetic parameters by the Michaelis-Menten (Equation 1).

$$v = \frac{[E]_0[S]k_{cat}}{K_m + [S]} \quad (1)$$

where [E]$_0$ is the concentration of the switch, [S] is the concentration of nitrocefin and k_{cat} and K_m are the Michaelis-Menten kinetic parameters. In the absence of maltose, the velocity is found by Equation 2

$$v^- = \frac{[E]_0[S]k_{cat}^-}{K_m^- + [S]} \quad (2)$$

where the superscript "-" designates that the parameters are those when maltose is not bound to the switch. In the presence of saturating concentrations of maltose (i.e. maltose is bound to all switches; this occurs at very high concentrations of maltose relative to the dissociation constant K_d for maltose), the velocity is found by Equation 3

$$v^+ = \frac{[E]_0[S]k_{cat}^+}{K_m^+ + [S]} \quad (3)$$

where the superscript "+" designates that the parameters are those when maltose is bound to the switch. At intermediate concentrations of maltose, the velocity depends on the fraction of switches that have maltose bound. If we make the approximation that the small cooperative effect of

maltose- and substrate-binding can be ignored, the fraction F of switches that are bound to maltose can be found by Equation 4.

$$F = \frac{[M]}{[K_d] + [M]} \quad (4)$$

where [M] is the concentration of maltose. The velocity of nitrocefin hydrolysis is thus found by Equation 5

$$v = F\frac{[E]_0[S]k_{cat}^+}{K_m^+ + [S]} + (1-F)\frac{[E]_0[S]k_{cat}^-}{K_m^- + [S]} \quad (5)$$

Equation 5 is true for all concentrations of maltose as it reduces to Equations 2 and 3 in the limiting cases of no maltose bound and saturating maltose, respectively. The fold-increase in the rate of nitrocefin velocity Z is found by dividing the right hand side of Equation 5 by the velocity in the absence of maltose to get Equation 6

$$Z = F\frac{k_{cat}^+\left(K_m^- + [S]\right)}{k_{cat}^-\left(K_m^+ + [S]\right)} + (1-F) \quad (6)$$

Equation 6 is plotted in Figure 1 for the case of RG13 hydrolysis of 25 µM nitrocefin using a range of different dissociation constants for maltose. One can see that the velocity is changing most in the range of one order of magnitude higher or lower than the dissociation constant for maltose. The switch will have the largest change in the dependent function if the concentration of the signal (in the case of RG13 this is maltose) changes within this range or changes through this range. Thus, it is desirable for the application of molecular switches to create switches with different affinities for the signal so as to be useful for different concentration ranges of the signal.

In one aspect of the invention, switches created by the method have different affinities for maltose. For example switch RG-5-169 (sequence MBP[1-338]-BLA[34-286]-GSGGG-BLA[24-29]-MBP[337-370] has a K_d for maltose (> 1 mM) that is much greater than RG13 has for maltose (1-5 µM).

In another aspect of the invention, the effector-affinity of switches is altered by a variety of methods including rational design and directed evolution methods. As long as the resulting altered-affinity switch maintains a conformational change upon binding the effector that results in changes the dependent function, switching will be maintained.

In another aspect of the invention mutations already known to alter the affinity of the ligand recognition domain (for RG13 this is MBP) are introduced into the switch to create switches with altered affinity for the ligand. These mutations consist of those that make direct contact with the ligand, those that make contact with residues that make direct contact with the ligand and those that are more distal from the binding site pocket. For example, Marvin and Hellinga (Marvin and Hellinga 2001) have described a set of mutations in the hinge region of MBP that manipulate the conformational equilibria between the open and closed state (Marvin and Hellinga 2001). Residual dipolar couplings have been used to establish that the apo forms of these mutants are partially closed relative to the apo wildtype MBP with the closure angles being 9.5° and 28.4° for I329W and A96W/I329W, respectively (the ligand-bound closed form of MBP has a closure angle of 35°)

(Millet, Hudson et al. 2003). Because partial closing shifts the equilibrium towards the ligand-bound state, the I329W mutation results in about a 20-fold increase in affinity for maltose and the A96W/I329W double mutant results in a 60-fold increase in the affinity for maltose compared to wildtype MBP at 25°C (Marvin and Hellinga 2001). The affinities of MBP, MBP(I329W) and MBP(I329W/A96W) are 800 nM, 35 nM and 13 nM, respectively. Introduction of these mutations into RG13 results in mutants with increased affinity for maltose (Table 1) while still maintaining switching behavior (Table 2 and Figure 2). In addition, the level of activity in the presence of saturating amounts of maltose (the "on" state) was not affected by the mutations (Table 1). From a practical standpoint, the increase in maltose affinity of these hinge mutants indicates that ligand-affinity of RG13 can be systematically changed to create molecular switches that respond to different concentration ranges of effector while still maintaining switching ability and high activity in the presence of the effector. By increasing the affinity for maltose one increases the sensitivity of the switch (i.e. it will switch to a higher level of activity at lower concentrations of maltose). In a further aspect of the invention, combinations of these affinity-altered switches behave as a composite switch with a high dynamic range.

The invention also consists of methods to alter the specificity of the signal recognition domain so that it recognizes other signals. This would allow the construction of new molecular switches in which the dependent function responded to new signals without requiring the construction of entirely new molecular switches. For the example of RG13, where the signal binding domain is the maltose binding protein, these methods can change the ligand to which the switch binds to. This would allow the construction of molecular switches in which BLA activity could be modulated by different ligands without developing entirely new molecular switches. Thus, in a further aspect of the invention, the identity of the signal to which the switch responds can be altered by mutations in existing switches. For example, mutations in the signal recognition domain already known to alter the ligand-binding specificity can be introduced into the switch to create switches that respond to new ligands. For example, Hellinga and colleagues have computationally designed periplasmic binding proteins with radically different binding specificities (Looger, Dwyer et al. 2003) including designing MBP to bind Zn^{2+} (Marvin and Hellinga 2001) instead of maltose. MBP binds maltose with high affinity ($K_d = 0.8$ μM) but does not bind Zn^{2+}. MBP with the A* set of mutations (A63H/R66H/Y155E/W340E) has high affinity for Zn^{2+} ($K_d = 5.1$ μM) and does not bind maltose (Marvin and Hellinga 2001). Introduction of the A* set of mutations into RG13 results in a switch that responds to Zn^{2+} but not maltose.

In a further aspect of the invention, the signal recognition domain is altered by rational design or directed evolution to bind to new effectors. With respect to testing mutations predicted by rationale design or screening or selecting libraries created for a directed evolution approach, existing switches are used to efficiently test or select for binding to new ligands *in vivo*. For example, *E. coli* cells expressing the MBP-BLA switch RG13 from the lac promoter on pDIMC8 have a higher MIC for ampicillin (Amp) in the presence of maltose than in their absence (Table 3) because the BLA enzymatic activity of RG13 (hydrolysis of ampicillin) is higher in the presence of maltose. Thus, for example, mutations created in RG13 (either by rational design or by a stochastic or semi-stochastic method) such that mutant forms of RG13 bind another ligand X (and behave as a switch) can be screened or selected for *in vivo*. *E. coli* producing such a new switch will grow at 200 μg/ml Amp in the presence of X but not in the absence of X.

In a further aspect of the invention, ligands that bind to the signal recognition domain in a different manner have different switching ability. This is demonstrated by the fact that

β-cyclodextrin, which is known to bind to MBP but with a different conformational change (Skrynnikov, Goto et al. 2000; Hwang, Skrynnikov et al. 2001), changes the activity of the RG13 switch in a different manner than maltose (see Table 2).

Table 1 Hinge Mutations in MBP domain Increase Maltose Affinity in RG13.

Protein	Ligand	K_d maltose $(\mu M)^a$			
		No Substrate[b]	25 μM nitrocefin[c]	Saturating Carbenicillin	
				IPF[b]	Enzymatic assay[d]
RG13	maltose	5.5 ± 0.5	6.7 ± 0.03	1.3 ± 0.5	0.9 ± 0.1
RG13 I329W	maltose	0.55 ± 0.13	1.0 ± 0.04	nd	0.11 ± 0.01
RG13 I329W/A96W	maltose	nd	0.17 ± 0.02	nd	nd

[a]Conditions: 100 mM NaCl, 50 mM $NaPO_4$, pH 7.0, 25°C

[b]Determined by measuring intrinsic protein fluorescence (IPF) as a function of maltose concentration. When using IPF at saturating carbenicillin, a concentration of 10 mM carbenicillin was used.

[c]Determined by measuring the initial rate of nitrocefin hydrolysis as a function of maltose concentration. 25 μM nitrocefin is well below the K_m of nitrocefin. Thus, most molecules of RG13 will not have nitrocefin bound and the effective K_d that is measured is close to what it would be in the absence of substrate.

[d]Determined by measuring the initial rate of carbenicillin hydrolysis as a function of maltose concentration. A concentration of 1.5 mM carbenicillin was used, which is well above the K_m of carbenicillin. Thus, most molecules of RG13 will have carbenicillin bound and the K_d that is measured is in the presence of bound substrate (carbenicillin).

Table 2 Kinetic parameters of nitrocefin hydrolysis[a].

Protein	Effector	k_{cat} $(s^{-1})^a$	k_{cat} Ratio[b]	K_m $(\mu M)^a$	K_m Ratio[b]	k_{cat}/K_m^a $(s^{-1} \mu M^{-1})$	k_{cat}/K_m Ratio[b]
RG13	-	200 ± 40	-	550 ± 120		0.37 ± 0.10	
RG13 I329W	-	190 ± 30	-	350 ± 60		0.54 ± 0.11	
RG13 I329W/A96W	-	360 ± 40	-	260 ± 40		1.4 ± 0.3	
RG13	maltose	620 ± 30	3.1 ± 0.6	68 ± 4	0.12 ± 0.03	9.2 ± 0.7	25 ± 7
RG13 I329W	maltose	590 ± 50	3.1 ± 0.5	53 ± 7	0.15 ± 0.03	11.0 ± 1.8	20 ± 5
RG13 I329W/A96W	maltose	530 ± 20	1.5 ± 0.2	60 ± 4	0.23 ± 0.04	8.9 ± 0.8	6.4 ± 1.3
RG13	β-cyclo[c]	590 ± 60	2.9 ± 0.6	870 ± 90	1.6 ± 0.4	0.67 ± 0.10	1.8 ± 0.6

[a]Conditions: 100 mM sodium phosphate buffer, pH 7.0, 25°C; concentration of effector is 5 mM
[b](with effector)/(without effector).
[c]β-cyclodextrin

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Table 3 Minimum inhibitory concentration of ampicillin for _E. coli_ cells expressing RG13[a]

Supplement to plate	MIC ampicillin (μg/ml)
none	100
50 μM maltose	400
5 mM maltose	400

[a]conditions: LB plates, 37 °C, supplemented with maltose as indicated. Approximately 1000 colony forming units (without ampicillin) per plate. Concentrations of ampicillin tested: 0, 25, 50, 100, 200, 400 and 800 μg/ml.

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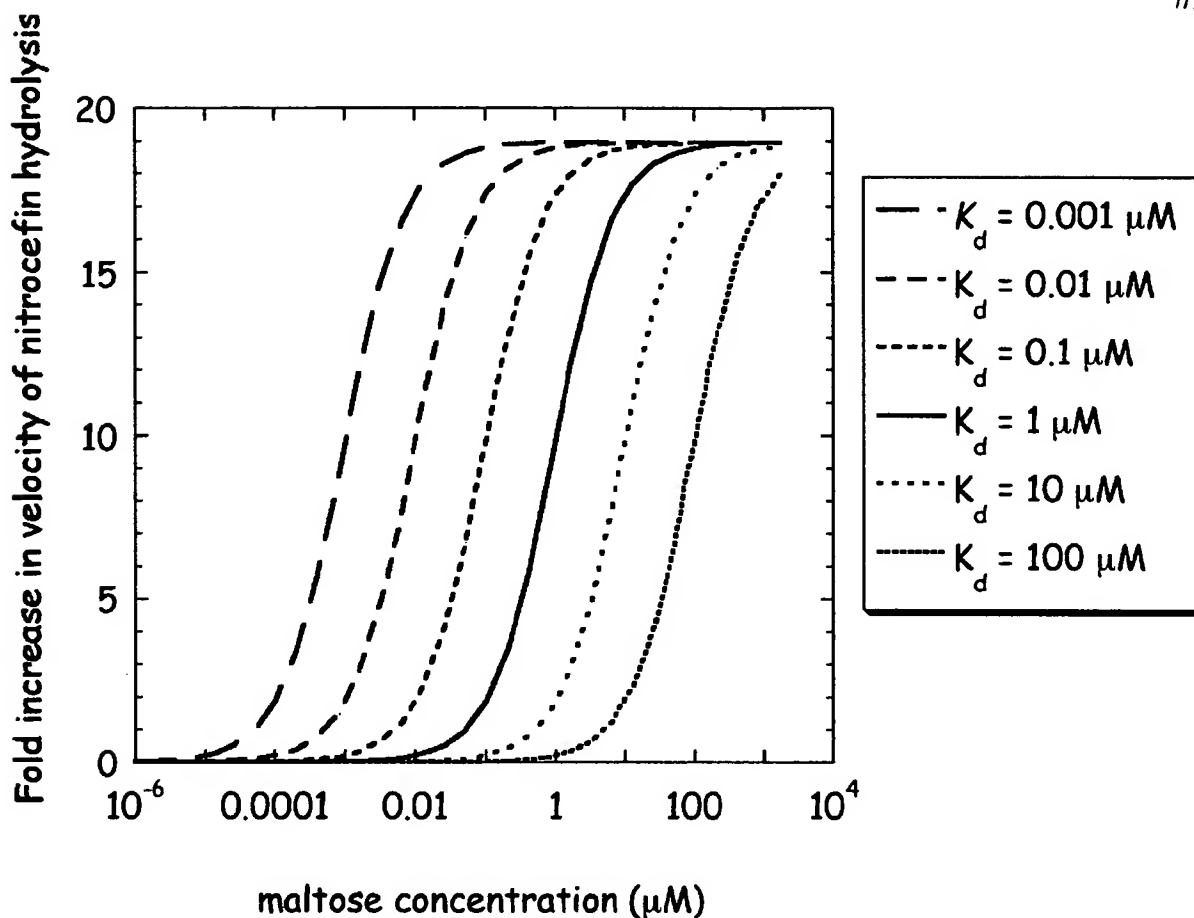
Figure 1 Fold increase in velocity of switch RG13 with different dissociation concentrations (K_d) for maltose. Concentration of nitrocefin was 25 µM. The kinetic parameters of RG13 with and without maltose are those in Table 2. Equation 6 was used to generate the curves.

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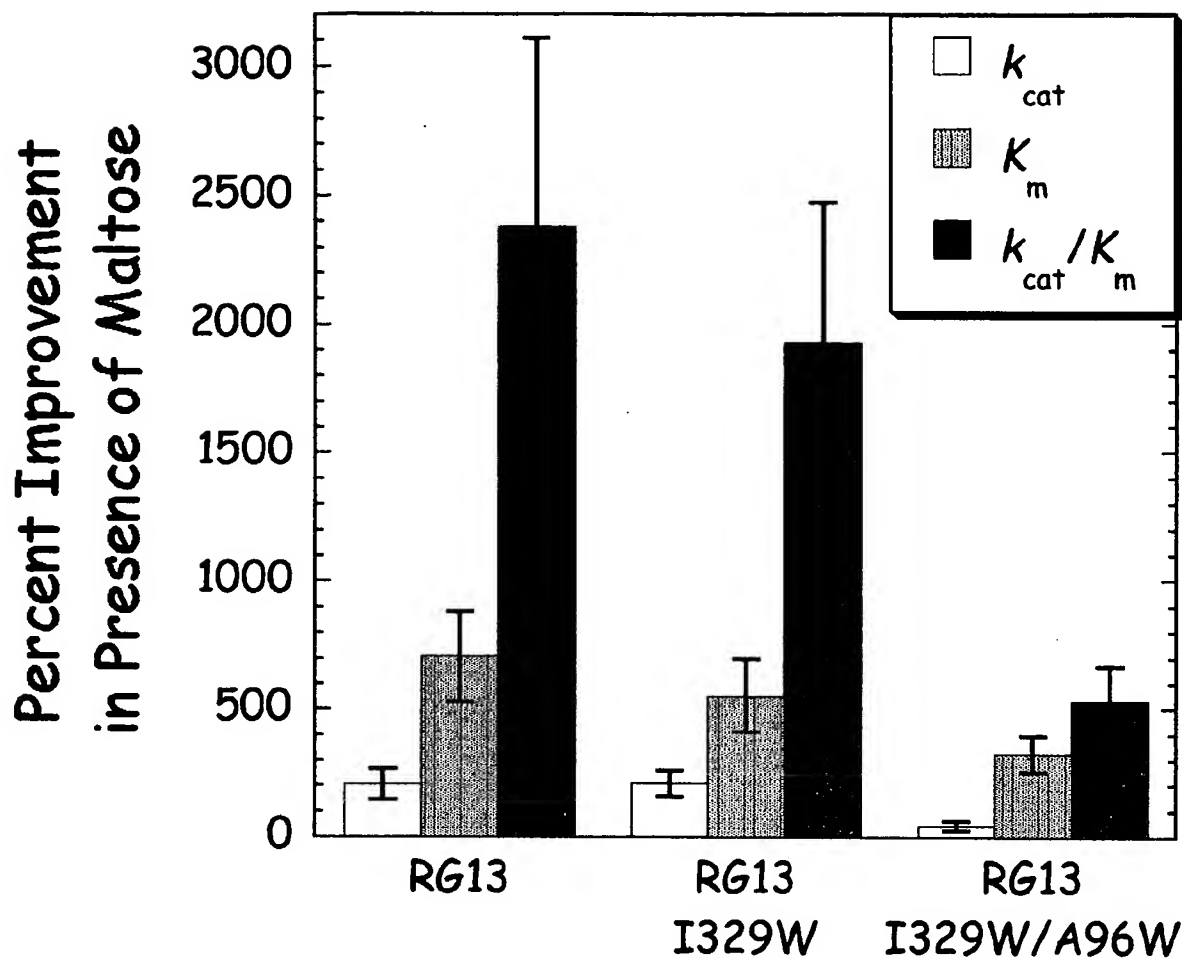
Figure 2. Percent improvement in kinetic parameters of nitrocefin hydrolysis of switches in presence of maltose. Data taken from Table 1. For k_{cat} and k_{cat}/K_m, an improvement is an increase in the value. For K_m, an improvement is a decrease in the value.

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